

Development of an ^{125}I -Postlabeling Assay as a Simple, Rapid, and Sensitive Index of DNA-protein Cross-links

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A rapid, simple, and sensitive ^{125}I -postlabeling technique has been developed to allow detection of DNA-protein cross-links induced by environmental contaminants and carcinogens. This method is based on specific incorporation of ^{125}I into tyrosine residues associated with DNA. Cultured Chinese hamster ovary cells were exposed to various crosslinking agents, e.g., UV light, K_2CrO_4 , or NiCl_2 . DNA was isolated by proteinase K/phenol/chloroform. The residual peptides cross-linked to DNA were radioiodinated with Na^{125}I and chloramine T. After repeated precipitation with ethanol, the radioactivity was determined. The ^{125}I method was compared with a ^3H -tyrosine prelabeling method and found to be of similar sensitivity. — Environ Health Perspect 102(Suppl 3):301–304 (1994).

Key words: chromium, UV, nickel, tyrosine, iodination

Introduction

DNA-protein cross-links (DPCs) are thought to be important genotoxic lesions induced by environmental contaminants and carcinogens (1,2). They are not easily repaired, and may impede cellular replication, transcription, or repair processes (1,2). Various methods have been developed for purification and detection of DPCs. Some of them, however, are plagued by problems related to high background, due to DNA isolation without proteinase digestion (1,2). Recently, we designed an effective and sensitive assay for residual amino acid–DNA cross-links following proteinase K digestion in Chinese hamster ovary (CHO) cells that were prelabeled with a radioactive amino acid (3). The assay has been used to measure DPC formation in tissue culture induced by nickel and chromium compounds. Unfortunately, this assay can only be used in systems where cellular proteins can be radiolabeled.

Iodine isotopes, especially ^{125}I , offer several advantages in postlabeling methods. Iodine can be incorporated into the aromatic side-chain of tyrosine residues with

relative ease, yielding a stable radiolabeled compound. Iodine may also substitute into other amino acids (e.g., histidine) but the reaction rate is much less than that for tyrosine. We and others have shown that tyrosine is the predominant amino acid cross-linked to DNA by cross-linking agents including γ -radiation and some transition metals (3–6). The short half-life (60 days) is an advantage in isotope disposal. The counting efficiency of ^{125}I is high. The isotopic abundance is higher for current preparations of ^{125}I (up to 100%), giving an effective labeling rate. The radiation emitted by ^{125}I is less penetrating than that of ^{131}I , thus presenting a reduced radiation hazard.

In this article, we describe a simple, rapid, and effective assay for ^{125}I -postlabeling and detection of DPCs induced by UV light, formaldehyde (HCHO), NiCl_2 , K_2CrO_4 and *cis*-diamminedichloroplatinum(*cis*-Pt) in cultured CHO cells.

Materials And Methods

Cell Culture and DNA Isolation

Chinese hamster ovary (CHO) cells were seeded at 4×10^6 cells in 150 mm dishes in 20 ml of complete α -minimal essential medium (α -MEM) containing 10% fetal bovine serum in an atmosphere of 5% CO_2 . After 20 hr, the medium was replaced by minimal salts/glucose maintenance medium (SGM) containing HEPES (50 mM, pH 7.2), NaCl (100 mM), KCl (5 mM), CaCl_2 (2 mM) and glucose (5 mM), to facilitate cellular uptake of metal ions and absorption of UV light. The cells were exposed to various concentrations of

NiCl_2 , K_2CrO_4 and *cis*-Pt for 5 hr and HCHO for 30 min as indicated in the figures. For UV light exposure, a wavelength of 254 nm, at a fluence of $2.8 \text{ J/m}^2/\text{sec}$ was applied with a General Electric Lamp (No. G15T8). The maximal dose of UV was 4000 J/m^2 .

DNA was isolated from CHO cells as previously described (3). Briefly, cells were scraped with a rubber policeman, collected and washed by repeated centrifugation and suspension. The cell pellet was lysed in 10 mM Tris, pH 8.0, containing 100 mM NaCl, and 0.5% SDS. RNase (10 mg/ml) was added, and a high concentration of proteinase K (500 mg/ml) was applied to hydrolyze the protein. The DNA pellet containing residual polypeptides and amino acids, was repeatedly extracted with phenol/chloroform, and the final aqueous layer was precipitated with ethanol.

^{125}I -Radioactive Postlabeling

^{125}I -postlabeling of residual polypeptides and amino acids associated with the DNA was performed by modification of the iodination technique described by Neuer et al. (7). Fifty micrograms DNA precipitated with ethanol was suspended in 100 ml of 2% SDS, 30% urea and 0.5M Tris-HCl, pH 7.6, and mixed with 10 mCi of Na^{125}I (17.4 Ci/mg) and 5 ml of chloramine T solution (6 mg/ml). For rapid and efficient iodination, reagents in the reaction mixture were highly concentrated. After 2 min at room temperature, the iodine was reduced by addition of 10 ml of 50% β -mercaptoethanol. DNA was collected together with the associated radiolabeled tyrosine by

This paper was presented at the Second International Meeting on Molecular Mechanisms of Metal Toxicity and Carcinogenicity held 10–17 January 1993 in Madonna di Campiglio, Italy.

This work was supported by grants ES 04895, ES 04715, ES 05512 and ES 00260 from the National Institute of Environmental Health Sciences, and by NIH grant CA 13343, Kaplan Cancer Center.

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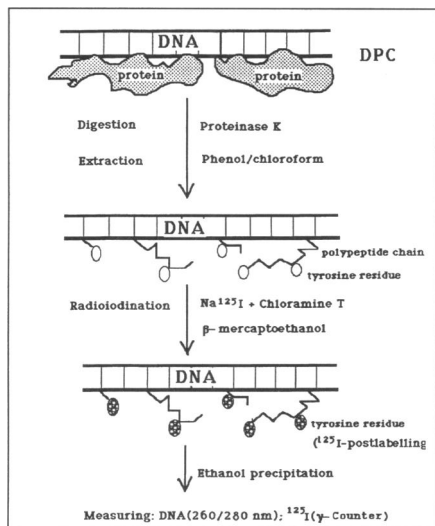


Figure 1. The ^{125}I -postlabelling protocol.

repeated (5 times) precipitation with ethanol and was finally dissolved in 10 mM Tris, pH 8.0. The unincorporated ^{125}I in the supernatant was washed off. The DNA samples were assayed for radioactivity in a γ -counter and the same samples were used for measurement of UV absorbance at 260/280 nm. ^{125}I labeling was expressed as cpm/mg DNA. The standard assay described above was optimized according to a series of experimental studies (Results).

To estimate the iodination efficiency, some cell samples were prelabeled with ^3H -tyrosine before exposure to the cross-linking agents. DPCs were analyzed as previously

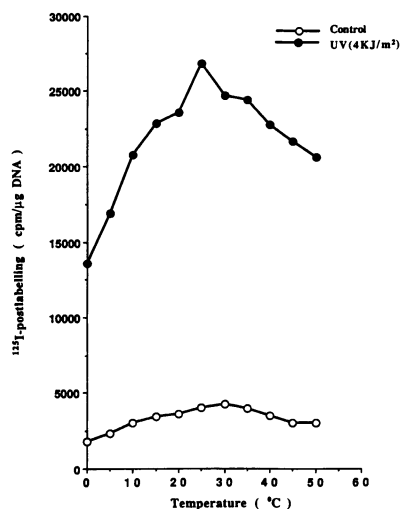


Figure 2. The effect of temperature on ^{125}I -postlabelling of residual peptides associated with DNA isolated from control and UV-exposed CHO cells. Iodination was conducted at the indicated temperatures, pH 7.5, 4 mCi/10 mg DNA for 5 min, after which samples were precipitated with ethanol in five cycles.

described (3), and the results were compared with that of the ^{125}I -postlabelling. (A scheme for the ^{125}I -labeling technique is shown in Figure 1).

Results and Discussion

Initially, experiments were performed to establish the optimal conditions for radioiodination of tyrosine residues associated with DNA in control and UV-treated CHO cells. Variables such as pH, temperature, reaction time, ethanol precipitation procedures, and ^{125}I /DNA ratios were optimized. As shown in Figure 2, chloramine T-mediated iodination was strongly temperature-dependent, and a maximum yield of iodine incorporation was achieved at 25°C to 35°C. Further increases of reaction temperature resulted in a decrease in iodination yield, which was probably due to increased side reactions at higher temperatures. Some investigators prefer to carry out iodination protocols on ice to reduce side reactions and oxidative damage induced by chloramine T; however, we found that iodination at temperatures less than 25°C also reduced the reaction rate. We suggest that iodination at room temperature is both suitable and convenient.

Figure 3 shows that the optimal pH value for iodination of tyrosine residues was 7.5. Above pH 8 there was a tendency toward decreased iodination yield, and above pH 9, the reaction became highly inefficient. Since the isotope Na^{125}I was usually supplied as a solution in 0.1 N NaOH, the composition of the other reactants must be modified to buffer the isotope to pH 7.5.

Figure 4 shows that chloramine T-mediated iodination of tyrosine residues was instantaneous, such that the minimum reaction time required to obtain a maximal yield of iodination at room temperature was 1 min. After this, the yield remained constant for 20 min. Incubation for much longer times caused oxidative damage to DNA and peptides, and consequently decreased the product yield. To ensure an adequate mixture of reactants, we chose 2 min as the standard incubation time.

The effects of ethanol precipitation on recovery of ^{125}I -labeled tyrosine residues is shown in Figure 5. Following iodination, the radiolabeled material could be precipitated from iodination buffer containing SDS, urea, and β -mercaptoethanol by ethanol, while unincorporated ^{125}I in the supernatant was washed off. After four or five precipitation cycles, the recovery of the radioactive label remained constant. Stable

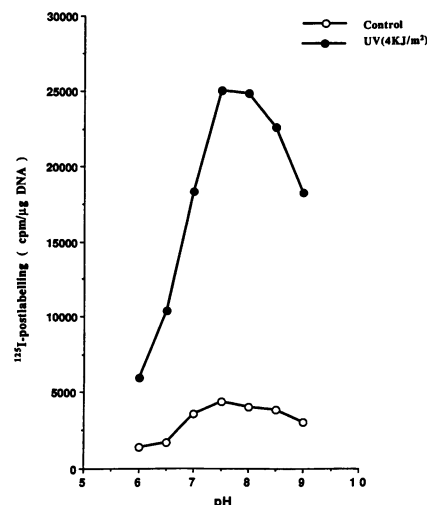


Figure 3. The effect of pH on ^{125}I -postlabelling of residual peptides associated with DNA isolated from control and UV-exposed CHO cells. Iodination was conducted at the indicated pH, at room temperature, 4 mCi/10 mg DNA for 5 min, after which samples were precipitated five times with ethanol.

radioactivity represented tyrosine residues crosslinked to DNA.

To establish the ratio of ^{125}I /DNA for optimal efficiency of radioiodination, the ratio of iodine to DNA in reaction mixtures was varied and the specific activity of the products was determined. The curve shown in Figure 6 demonstrates that the optimal yield of radioiodination is achieved at iodine to DNA ratios of 1.5 and 2.0

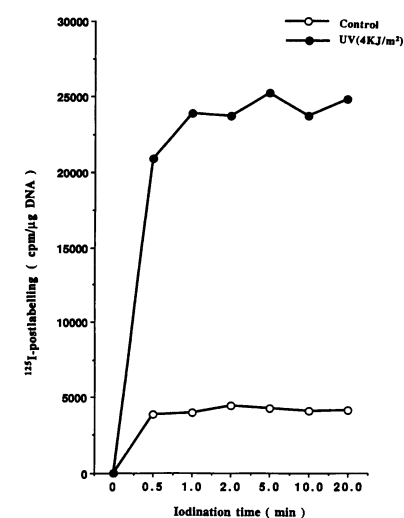


Figure 4. The effect of iodination time on ^{125}I -postlabelling of residual peptides associated with DNA isolated from control and UV-exposed CHO cells. Iodination was conducted at room temperatures, pH 7.5, 4 mCi/10 mg DNA for the indicated intervals, and then samples were precipitated five times with ethanol.

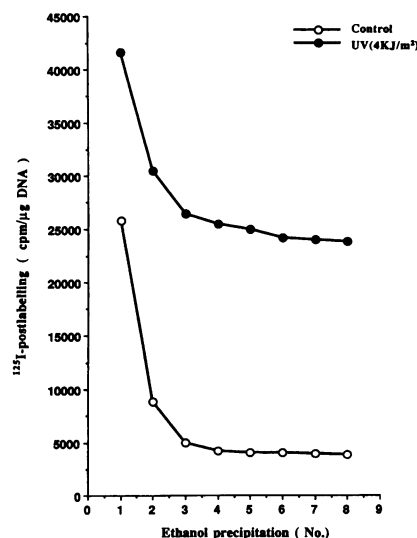


Figure 5. The effect of ethanol precipitation on ^{125}I -postlabeling of residual peptides associated with DNA isolated from control and UV-exposed CHO cells. Iodination was conducted at room temperatures, pH 7.5, 4 mCi/10 mg DNA for 5 min, then samples were precipitated with ethanol for the number of cycles indicated.

mCi/10 mg for control and UV-treated CHO cells, respectively. Further increases in this ratio did not cause any increase in the specific radioactivity of the resulting reaction products. This means that under these reaction conditions, all the iodine incorporation sites in tyrosine residues associated with DNA have been saturated.

Using the standard conditions estab-

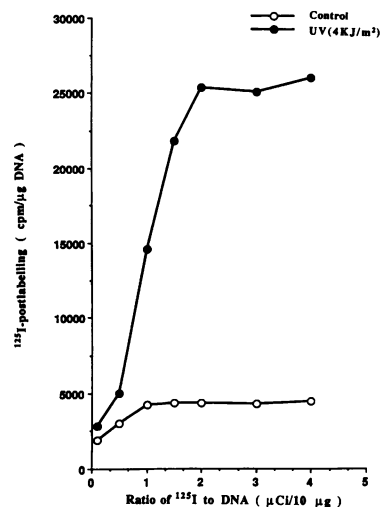


Figure 6. The effect of temperature on ^{125}I -postlabeling of residual peptides associated with DNA isolated from control and UV-exposed CHO cells. Iodination was conducted at indicated ratios of ^{125}I /DNA at room temperature, pH 7.5, for 5 min, and then the samples were precipitated with ethanol.

lished above, we examined DPC formation in cultured CHO cells induced by UV, HCHO, chromium(VI), nickel(II), and *cis*-Pt agents which have been demonstrated by other studies to yield DPCs by quite different mechanisms (2). UV light produces radicals in the ring structure of DNA bases, which then react with adjacent proteins to form covalent bonds (8). HCHO forms covalent bonds between the S amino groups of lysine residues and adjacent DNA bases (9). Chromium(III), the reduced form of chromium(VI), and the dechlorinated form of *cis*-Pt have an affinity for histidyl, methionyl, and cysteinyl residues of proteins and the nitrogens or phosphates of DNA bases (10). Nickel(II) was thought to form stable protein-nickel(II)-DNA complexes, and a strong interaction between nickel(II) and amino terminal residues and imidazole group of histidine residue was demonstrated (11). On the other hand, increasing evidence suggests that some transition metals may generate reactive oxygen species (ROS), which may indirectly mediate DNA damage, protein oxidation, and DPC formation (12,13). Regardless of the mechanisms by which DPCs are formed, increasing levels of DPCs were likely to affect the structure and function of DNA. It is noteworthy that nucleoprotein in normal cells, as an important component for maintenance of DNA conformation, replication, and transcription, is also closely associated with DNA by phosphotriester bond between internucleotide phosphate or other unknown bonds. The presence of these nucleoproteins results in higher background and lower sensitivity of most DPC assays. To solve this background problem, we previously developed a new method for analysis of residual amino acid-DNA cross-links as an index of DPC formation after prelabeling CHO cells with radioactive amino acid and isolation of DNA by standard proteinase K/phenol extraction (3). This method has been demonstrated to be highly sensitive for isolation of DPCs from chromium(VI)- and nickel(II)-treated CHO cells (3). The assay presented here has obvious advantages over the previous method, in which the prelabeling with a radioactive amino acid is substituted by ^{125}I -postlabeling of tyrosine residue associated with DNA. Figure 7 illustrates that all five test agents induced increased ^{125}I -labeling efficiency in a dose-dependent manner. Particularly significant increases were noted for UV light and chromium(VI). With nickel(II), a smaller and significant increase of ^{125}I -labeling was

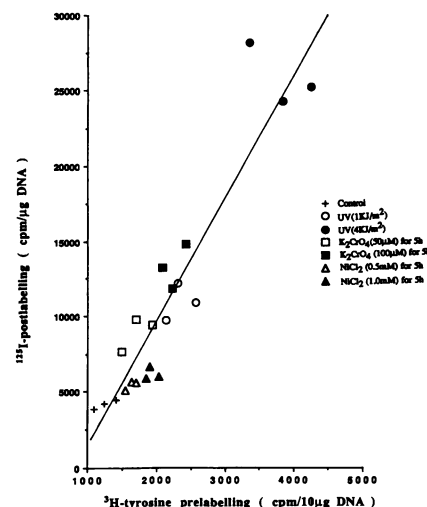


Figure 7. The use of the ^{125}I -postlabeling assay for determination of DPCs formed in CHO cells by some cross-linking agents. CHO cells were exposed to various concentrations of the indicated agents. Following these treatments, residual peptide-DNA complexes were isolated by proteinase K/phenol/chloroform extraction. ^{125}I -postlabelling was conducted as described in Materials and Methods. Each point presents the mean \pm SEM from three separate samples.

shown. In a comparison between the post-labeling and prelabeling protocols with CHO cells exposed to UV, chromium(VI)

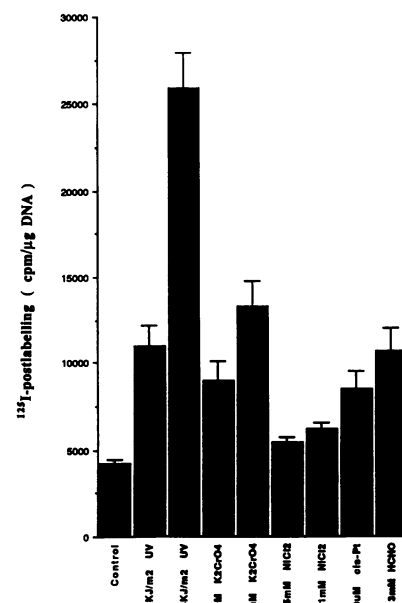


Figure 8. The correlation between ^{125}I -postlabeling and ^3H -tyrosine prelabeling assays. CHO cells were prelabelled with ^3H -tyrosine for 24 hr prior to exposure to indicated agents. DNA was isolated by proteinase K/phenol/chloroform extraction. Residual tyrosine complexed with DNA was determined as previously described (3). The results were compared with that of the ^{125}I -postlabeling method.

and nickel(II), a significant correlation between the two assays is found (in Figure 8, $r=0.864$, $p<0.01$). In fact, it appears that the postlabeling method is more sensitive than prelabeling assay. The ^{125}I -post-labeling assay allows the sensitive detection

of DPCs in human samples from individuals exposed to environmentally adverse agents.

In summary, ^{125}I -postlabeling technique can be used to detect DPC formation. It can be applied to experimental

studies and can be used in risk assessment and epidemiologic investigations of human population exposed to environmental carcinogens or mutagens.

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